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Reconstitution of Functionalized Transmembrane Domains of Receptor Proteins into Biomimetic Membranes

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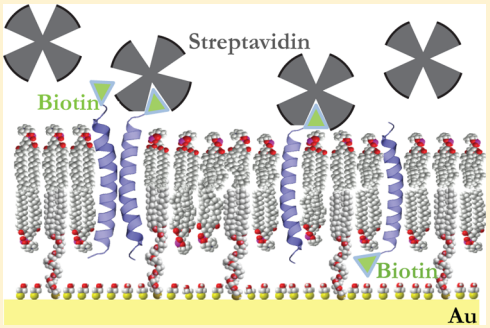
Supporting Information

ABSTRACT: For integral membrane proteins, an assessment of their structures and interactions within a biomimetic lipid bilayer environment is critical for evaluating their cellular function. Hydrophobic sequences prevalent within transmembrane domains, however, make these proteins susceptible to aggregation and, thus, create difficulties in examining their structural and functional properties via canonical techniques. Working exclusively with single-pass transmembrane (TM) segments of bitopic membrane proteins, in the form of soluble peptides, bypasses many of the pitfalls of full-length protein preparations while allowing for the opportunity to examine the properties of TM domains within biomimetic membrane environments. In this study, peptides mimicking the TM domains of the epidermal growth factor receptor (EGFR) and CD4 co-receptor, both cell-signaling surface receptors, have been reconstituted into 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipid bilayers. The formation of their native α-helical structures within vesicle membranes was observed from circular dichroism, and full partition of the peptides into the membrane was demonstrated by tryptophan fluorescence and neutron reflectivity (NR). Using an engineered planar lipid bilayer system ideal for surface characterization methods, such as surface plasmon resonance (SPR) and NR, the TM peptides, functionalized with a N-terminal biotin tag, proved capable of "activating" a membrane surface, as evidenced by the capture of streptavidin. On the basis of these initial assessments, we anticipate these membrane-bound peptides will provide a versatile platform for understanding the intricate roles of receptor TM domains in cell signaling.



INTRODUCTION

Proteins embedded within biological membranes, referred to as integral membrane proteins (IMPs), participate in many vital processes, such as cell proliferation, differentiation, adhesion, signal transduction, and substrate trafficking. Understanding the mechanisms by which membrane proteins operate within their cellular framework is critical for finding preventative diagnostics and effective treatments for the diseases caused by IMP malfunction.[1,2] Biochemical and structural studies of cell surface receptors, a class of IMPs that frequently functions synergistically with other membrane or peripheral-binding proteins, have revealed that transmembrane (TM) domains have significant parts to play in the relaying of signals across the membrane.[3,4] Indeed, the single-spanning TM domains of related receptors are commonly known to interact and participate in the formation of higher ordered receptor complexes with a heightened capability of triggering an intracellular response.[5−8]

Still, the level of participation of the TM domain in propagating signals through the membrane, itself a dynamic component that could mediate the stabilization of an active receptor complex, remains difficult to interpret from biochemical and structural techniques. Thus, investigating structural features of TM domains of receptor proteins within lipid bilayers and the process by which they organize, by means of underutilized surface characterization methods and a biomimetic lipid bilayer system, could help resolve the critical mechanisms of cell-signaling transduction.

Efforts toward sequestering membrane proteins in biomimetic lipids have led to the development of novel protein preparations (*in vitro* cell-free expression,[9] co-expression with detergents,[10] nanodiscs,[11] etc.), in conjunction with artificial membrane scaffolds (soluble lipid vesicles, preformed membranes, lipid mesophases, etc.).[12,13] Namely, tethered bilayer lipid membranes (tBLMs) are solid-supported, two-dimensional phospholipid systems that can be fabricated to closely mimic the fluid and chemically heterogeneous environment of the biological membrane[14,15] and are well-established for investigating both peripheral and integral membrane protein interactions with a bilayer.[16−20] Delivering IMPs into the tBLMs has been performed by a number of methods, from directly synthesizing the protein in the presence of the tBLM *in vitro*[9] to transferring expressed and refolded proteins from membrane-mimetic surfactants, detergents, or amphipols.[10,12,13] As a reductionist approach, synthesizing peptides comprised mainly of the TM domain of model receptor proteins offers a less complicated method for membrane deposition and

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biophysical characterization of integrated TM domains of model surface receptors. There is a wealth of data supporting that TM α-helices of IMPs can be treated as autonomous folding domains and, thus, can be evaluated within membrane mimetic environments independently from their extra-membrane domains.[21−25] Recent advances in engineering water-soluble peptide analogues has greatly facilitated the study of TM-centric organization of membrane proteins in detergent micelles and lipid vesicles.[26,27] For instance, solubility of the epidermal growth factor receptor (EGFR) TM peptide in an aqueous environment was accomplished by capping both termini of the peptide with lysine residues, to reduce the hydrophobicity of the overall peptide below a calculated threshold while keeping the TM domain intact.[28]

In this study, we have adopted a similar peptide design, with additional modifications for assessing membrane incorporation and functionalization, which can ultimately be used for reconstituting membrane proteins into soluble vesicles and planar lipid bilayers. Soluble TM peptides of EGFR, along with the CD4 T-cell co-receptor, were synthesized with a biotin moiety at the N terminus and a tryptophan residue within the TM sequence. The EGFR and CD4 TM peptides were shown to partition into 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipid vesicles, with a significant population of the TM domains adopting their native α-helical conformations, evidenced by tryptophan fluorescence and circular dichroism (CD). Next we set out to anchor the TM peptides within a tBLM system, comprised of POPC lipids and tethered to a gold-coated surface. Association of the peptide to the planar membrane resulted in selective capture of streptavidin (SA) through the exposed biotin moiety, as observed by surface plasmon resonance (SPR) measurements. Correspondingly, the profile of the TM peptides and bound SA along the membrane normal direction could be structurally modeled using neutron reflectivity (NR).[29] By characterization of the insertion and localization behavior of these functionalized TM domains, this work showcases the potential of membrane−peptide systems for evaluating a range of modified cell surface complexes.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were commercially synthesized by PrimmBiotech, Inc. (Cambridge, MA) with >95% high-performance liquid chromatography (HPLC) purity, and their weights were verified by matrix-assisted laser desorption/ionization time of flight (MALDI−TOF). The sequence of the CD4 TM peptide (biotin-KKKKKMALIVLGGVAGLLLWIGLGIFFSKKKK-$CONH_2$) includes the estimated TM domain (underlined) as predicted by the TM Finder program.[30] The EGFR (biotin-KKKKIATGMVGALLLLWVVALGIGLFMKKKK-$CONH_2$) and $E_3\alpha$ (biotin-KKAAAEAAAAAEAAWAAEAAAKKKK-$CONH_2$; no predicted TM domain) peptides are adopted from previous investigations of TM folding,[27,28] with the appropriate modifications for our ligation and fluorescence studies. Further description of our peptide design is included in the Supporting Information.

Lipid Preparations. POPC lipids pre-dissolved in chloroform were purchased from Avanti Polar Lipids (Alabaster, AL), and aliquoted into vials for freeze-dry evaporation of the solvent. For fluorescence and CD experiments, vesicle stock solutions of 5 mmol/L (≈mM) were made in an aqueous Tris buffer (10 mM Tris−HCl and 10 mM NaCl with a volume fraction of 10% acetonitrile at pH 7.2) and resuspended via bath sonification. Brominated lipids 1-palmitoyl-2-[*m*,*n*-dibromo]stearoyl-*sn*-glycero-3-phosphocholine ("*m*,*n*-Br_2PC") dissolved in chloroform were mixed to 1:1 molar ratios with POPC before freeze-drying. For SPR and reflectivity measurements, lipids were dissolved in anhydrous-grade ethanol (Sigma-Aldrich, St. Louis, MO) to a final concentration of 10 mM.

Tryptophan Fluorescence. Peptides in stock solutions of water were aliquoted to a final peptide concentration of 5 μmol/L (≈ μM) in Tris buffer, with or without 1 mM POPC. Peptide samples mixed with POPC were sonicated for approximately 2 min and then incubated for approximately 1 h at 37 °C before taking measurements. Emission scans between 290 and 420 nm (at 1 nm intervals) were taken using a FluoroMax-3 fluorimeter (Horiba Jobin Yvon, Edison, NJ) with the chamber regulated at 37 °C. Samples of Tris buffer and 1 mM POPC in Tris buffer were measured for the purpose of blank subtraction. The reported values for quenching by the brominated lipids are normalized by the fluorescence intensities of peptide in native POPC (F_{Br}/F_{native}) and along with the blue shift ($\Delta\lambda_{max}$) values are averages of triplicate measurements (±standard deviation).

CD. Following a 1 h 37 °C incubation period, samples of peptides (20 μM) in Tris buffer, with either 15 mM sodium dodecyl sulfate (SDS) or 2 mM POPC, were placed in 0.1 mm demountable quartz spectrophotometer cells (Starna Cells, Inc., Alascadero, CA). For the mixtures of peptide with POPC vesicles, excess peptide was removed by overnight dialysis at 37 °C. Absorbance scans were performed on a Chirascan CD spectrometer (Applied Photophysics, Ltd., Leatherhead, U.K.) between 190 and 260 nm, collecting for the duration of 10 s per data point (at 1 nm interval readings). At least three independent samples were measured for each peptide. The instrumentation software (Pro-Data Software Suite) was used to subtract the background of the buffer, SDS, and lipid vesicles as necessary and conversion of the spectra to mean residue ellipticity (MRE; Θ = deg cm^2 $dmol^{-1}$). Modified spectra were uploaded to the DICHROWEB online server (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml)[31,32] to derive α-helicity character of the peptides. Additional details of the CD spectra fitting procedures using DICHROWEB are included in the Supporting Information.

SPR. All binding measurements were performed on a home-built SPR instrument, using a Neo 5.5 sCMOS camera (Andor Technology, Ltd., Belfast, U.K.). Simultaneous electrochemical impedance spectroscopy (EIS) measurements were performed using a saturated silver−silver chloride MI-401F reference electrode (Microelectrodes, Inc., Bedford, NH) and a Modulab Potentiostat (Solartron Analytical, Farnborough, U.K.). Substrate preparation and tethering self-assembled monolayer (SAM) formulation procedures are described in the Supporting Information.

Tethered lipid bilayers of POPC were formed by the rapid solvent exchange technique,[33,34] where the Au/SAM surface is exposed to ethanol solutions of POPC lipids (10 mM) for 2 min, and then the surface is flushed profusely with phosphate buffer (10 mM NaH_2PO_4 and 100 mM NaCl at pH 7.3). Successful formulation and stability of a tethered bilayer lipid membrane (tBLM) was confirmed by EIS measurements throughout the SPR experiments, with capacitance plots and fitted values provided in Figure S1 and Table S1 of the Supporting Information, respectively. Following the initial bilayer formation, lipid overlayers were removed by mild rinsing of the tBLM with ethanol solutions at a volume fraction of 25% in water, followed by thorough flushes with phosphate buffer. The neat bilayer was then incubated in Tris buffer, followed by incubations of 5 μM CD4, EGFR, or $E_3\alpha$ peptide for 5−10 min. A subsequent wash with Tris buffer was carried out to remove potentially non-associated peptide. Lyophilized SA (Life Technologies, Grand Island, NY) was dissolved in phosphate buffer and incubated with the membrane surface (±peptide) at a final concentration of 5 μM (or 20 μM when premixed with 5 μM EGFR TM peptide) for 5−10 min.

To determine the amount of protein associated with the tBLM surface, we calculated the surface coverage (reported as a percentage) from the change in pixels after protein binding. A conversion factor of 0.647 Å/pixel derived from calibration of the SPR instrument allows for the final calculation of the percent surface coverage of captured SA, along with the one-dimensional thickness of tetrameric SA (reported between a range of 43 and 48 Å).[35,36] Additional details and analysis procedures are provided in the Supporting Information.

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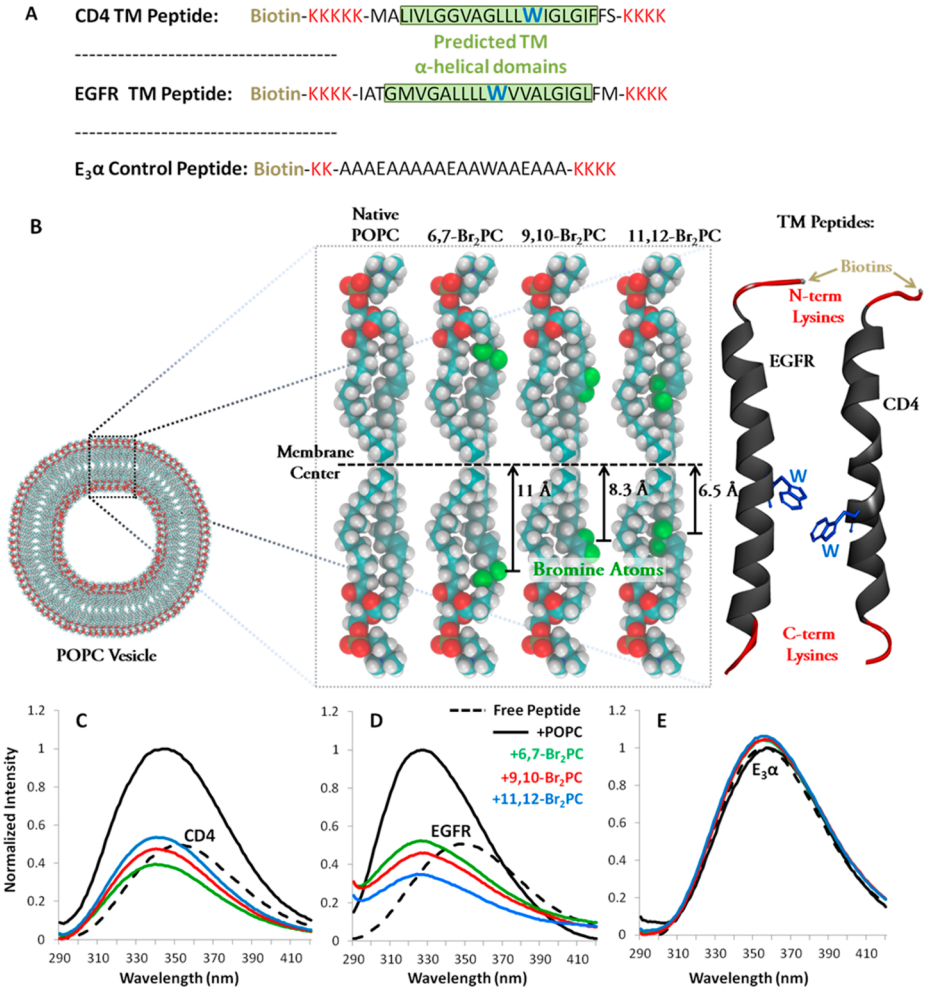

Figure 1. Insertion of model TM peptides in phospholipid vesicle membranes. (A) Peptide sequences and modifications. (B) Biotinylated and Trp-incorporated CD4 and EGFR TM peptides [generated from Protein Data Bank (PDB) codes 2KLU and 2M0B using Discovery Studio Visualizer, Accelrys Software, Inc., San Diego, CA] are depicted as normal to and centered within the bilayer of a lipid vesicle. The bromine atoms of the various brominated lipids used for fluorescence quenching are illustrated green, with their average distances to the membrane center.[40] Representative tryptophan fluorescence data of (C) CD4 TM, (D) EGFR TM, and (E) $E_3\alpha$ peptides in the absence (dotted black) and presence (solid black) of POPC vesicles, at 1:200 peptide/lipid molar ratios. All measurements were taken at 37 °C in Tris buffer. Curves representing POPC vesicles spiked with a mole fraction of 50% brominated lipids are colored according to the position of the bromine substitutions along the hydrocarbon chain of the lipid.

NR Measurements. A silicon wafer of 3 in. (7.6 cm) diameter, 5000 μm thick n-type Si:P[100] with one side polished to <5 Å roughness, was purchased from El-Cat (Waldwick, NJ). The Cr/Au/SAM surface was prepared similarly to the SPR procedures (see the Supporting Information for details). The wafer was then assembled into a standard NIST Center for Neutron Research (NCNR) reflectometry flow cell, and a POPC membrane was deposited by the rapid solvent exchange technique. NR was performed on the NG7 horizontal reflectometer and the MAGIK vertical reflectometer at the NCNR. A momentum transfer, q_z, range between 0.008 and 0.258 Å^{-1} was accessed in most measurements. Typical measurements used three solvent isotopic contrasts per experimental condition, consisting of aqueous Tris or phosphate buffer prepared in either 100% D_2O or H_2O or 67% D_2O/33% H_2O (CM4).

Parameter fitting was performed with the Refl1D software package (http://www.reflectometry.org/danse/docs/refl1d/index.html). All NR data sets collected at each stage of the experiments including all solvent contrasts are simultaneously fit conserving substrate parameters that are unaltered by the experimental conditions. Refl1D uses a Monte Carlo Markov Chain algorithm to robustly explore parameter space and provides confidence intervals in the membrane and protein volume profiles.[37] Further description of the protein modeling is provided in the Supporting Information.

RESULTS

Design of the TM Peptides for Structural Studies. To achieve our primary goal of anchoring single-span TM domains into a biomimetic membrane environment, water-soluble peptides mimicking the TM segments of the human CD4 co-receptor and the EGFR protein were designed and synthesized for structural examination (Figure 1A). Critical for this work in aqueous conditions, both TM peptides were capped with four or five lysines on both ends to effectively offset the hydrophobicity of the TM segments. Lys-capping is a procedure previously established in studies of the native EGFR TM domain, which report the folding of the peptide into its native α-helical structure when placed in a pseudo-lipidic environment of SDS micelles.[28] Another well-characterized, Lys-capped synthetic peptide with three glutamic acid residues interspersed within a hydrophobic sequence, referred to here as "$E_3\alpha$", has been adopted in our work as a negative

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Table 1. Blue Shifts[a] and Fractional Fluorescence Quenching[b] of TM Peptides into Lipid Vesicles

	CD4 TM		EGFR TM		$E_3\alpha$	
vesicles	$\Delta\lambda_{max}$ (nm)	F_{Br}/F_{native}	$\Delta\lambda_{max}$ (nm)	F_{Br}/F_{native}	$\Delta\lambda_{max}$ (nm)	F_{Br}/F_{native}
native POPC	−12 ± 7	N/A	−19 ± 4	N/A	2 ± 1	N/A
6,7-Br_2PC	−15 ± 4	0.39 ± 0.01	−19 ± 4	0.47 ± 0.08	1 ± 2	1.05 ± 0.05
9,10-Br_2PC	−13 ± 3	0.43 ± 0.06	−17 ± 4	0.44 ± 0.05	1 ± 2	1.07 ± 0.08
11,12-Br_2PC	−14 ± 4	0.49 ± 0.07	−18 ± 6	0.36 ± 0.01	0 ± 1	1.06 ± 0.05

[a]Blue shift values were determined by subtracting the maximum wavelength (λ_{max}) of the free peptide from that of the peptide in 1 mM POPC. [b]Fluorescent quenching caused by the 50% Br_2PC vesicles is relative to the intensity measured for peptides in native POPC. Standard deviations are provided for the average of three measurements for each lipid condition.

control for insertion into POPC membranes.[27] Conveniently, the $E_3\alpha$ peptide has a strong propensity to form an α-helical structure in aqueous solution; therefore, it also represents a positive control for secondary structure formation. Each of the three peptides was engineered with a N-terminal biotin and an internal tryptophan (Trp) substitution to allow for the comparative structural and functional assays.

TM Peptides of CD4 and EGFR Spontaneously Insert into POPC Vesicles. For this investigation, the Trp substitution in the TM sequence of the model peptides served as a direct probe of the insertion behavior into phospholipid vesicles. The emission maximum (λ_{max}) of tryptophan "blue shifts" toward lower wavelengths when exposed to a more nonpolar microenvironment, such as the hydrocarbon chains of a membrane bilayer. Therefore, to assess the partitioning of the TM peptides into lipid vesicles, steady-state Trp fluorescence spectra were measured for the peptides in the absence and presence of vesicles composed of POPC lipids.

Fluorescence measurements of all three peptides in 10 mM Tris buffer (10 mM Tris−HCl at pH 7.2 and 10 mM NaCl with a volume fraction of 10% acetonitrile to ensure that the peptides were fully soluble) produced spectra that possessed intensity maxima near 350 nm (panels C−E of Figure 1), corresponding to Trp fully exposed to an aqueous environment.[38] Upon mixing with POPC vesicles at a 1:200 molar ratio of peptide/lipid, the spectra for both the CD4 and EGFR TM peptides showed clear indications of insertion into the hydrophobic environment of the vesicles, with distinct blue shifts of 12 and 19 nm, respectively (Table 1). Concurrently, an increased intensity maxima was observed for both TM peptides, typical of Trp protected within lipid vesicles.[26,39] In comparison, the negative control peptide $E_3\alpha$ produced a negligible effect in fluorescence upon mixing with POPC (Figure 1E and Table 1), an expected result in light of previous reported measurements of the peptide in zwitterionic L-α-lysophosphatidylcholine (LPC) micelles.[27]

To best approximate the orientation of the TM peptides in relation to the associating vesicles, dibrominated phospholipids were introduced into the vesicles at a mole fraction of 50% (1:1 "native" POPC/m,n-Br_2PC). The two vicinal bromine substituents at the m and n positions within the fatty acid chains act as distance-dependent Trp quenchers[40] and can therefore be used to monitor the penetration of the internal Trp residues of the peptides within the vesicle bilayer. Using three brominated lipids of varying substituent positions (6,7-, 9,10-, and 11,12-Br_2PC), Trp fluorescence of both EGFR and CD4 was observed to change as a function of the bromine position, confirming that the two TM peptides were partitioning into the bilayer in a defined orientation. Figure 1B shows the relative positions of the bromine substituents within a vesicle bilayer and the possible orientation of a partitioning model TM peptide for context. Specifically, the EGFR TM peptide experienced the highest level of fluorescence quenching in the presence of the 11,12-Br_2PC vesicles (0.36 relative fluorescence remaining, F_{Br}/F_{native}), as opposed to the 9,10- and 6,7-Br_2PC vesicles (0.44 and 0.47 fluorescence remaining, respectively). Because the 11,12-Br_2PC lipids form vesicles with their bromine atoms positioned closest to the bilayer center, the Trp residue of the EGFR peptide would appear to reside proximal to the membrane center. Alternatively, fluorescence of the CD4 TM peptide was quenched most effectively in the presence of the 6,7-Br_2PC vesicles (0.39 fluorescence remaining), suggesting that its Trp residue is positioned more proximal to the lipid headgroups. This correlates with the Trp being closer to the C-terminal Lys residues in the CD4 peptide than for the EGFR peptide (refer to the primary sequences in Figure 1A). In stark contrast to both the TM peptides, fluorescence of the $E_3\alpha$ peptide is not diminished by any of the three varieties of brominated lipids, maintaining equivalent levels of intensity to that of the peptide in the presence of native POPC vesicles.

It is worth noting that the mole fraction of 50% brominated lipid vesicles produced the same level of blue shift fluorescence change as that of the native POPC vesicles when associated with the TM peptides (between 13 and 19 nm; Table 1). This suggests that the peptides interact with the brominated lipids similarly to the native lipids, and thus, their performance in distance-dependent quenching is an accurate determinant of peptide penetration of the CD4 and EGFR TM peptides in the POPC vesicles.

Structural Transitions of TM Peptides Observed Using CD. Because the native conformation of single-pass receptor TM domains is suspected to be α-helical in the plasma membrane,[41] it is reasonable to expect that the insertion of the TM peptides into POPC vesicles induces α-helical conformation. CD spectroscopy was performed to verify the secondary structure of the CD4 and EGFR TM peptides, along with the $E_3\alpha$ peptide for comparison. Spectra scans of the three peptides, initially in only Tris buffer, were collected to monitor their changes in structure upon the addition of POPC vesicles (panels A−C of Figure 2). As expected, the characteristic minima near 208 and 222 nm indicate that only the $E_3\alpha$ peptide is predisposed toward helical structure in the aqueous Tris solution. In contrast, the spectra of the EGFR and CD4 TM peptides indicate predominantly unordered secondary structure in Tris buffer. However, a significant transition toward α-helical structure occurs in the spectra profiles after introducing POPC vesicles to either the EGFR or CD4 peptides. Meanwhile, the CD profile of the $E_3\alpha$ peptide in the presence of POPC vesicles changed only slightly in magnitude from that in Tris buffer but not overall shape. Additional measurements of the TM peptides in SDS micelles provide a comparative assessment of the

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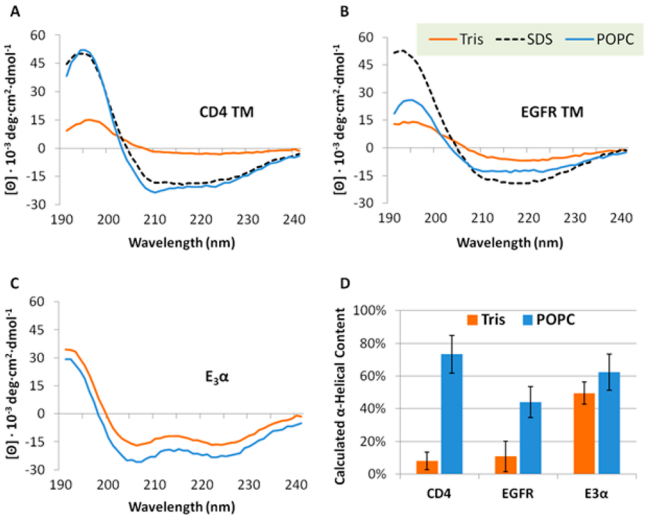

Figure 2. Secondary structure of peptides in aqueous and membrane-mimetic environments. (A) CD4 TM, (B) EGFR TM, and (C) $E_3\alpha$ peptides in Tris buffer, each at 20 μM, were measured for helical formation in the presence of 15 mM SDS micelles or 2 mM POPC vesicles. The far-ultraviolet (UV) CD spectra scans represent the molar residue ellipticity, Θ, of the peptides after buffer subtraction. (D) Percent α-helical character of the peptides in aqueous buffer or in the presence of POPC vesicles, as calculated from the online web server DICHROWEB.[31,32] Values are the averages of three to four independent measurements, and the standard deviations of the calculated values are indicated as error bars.

peptide conformations in a pseudo-lipidic environment. The spectra of the two TM peptides in POPC resemble spectra for the peptides in SDS micelles (more so for the CD4 peptide), showing that they adopt equivalent secondary structures in both detergent micelles and lipid vesicles.

As a more accurate means of analyzing the CD data, the DICHROWEB analysis Web server was employed to deconvolute the helical composition of the peptides in the presence and absence of POPC. DICHROWEB processes the experimental CD spectra against reference sets of spectra for proteins with known secondary structures using fitting algorithms. In agreement with the visual interpretation of the CD spectra, it was determined that the EGFR and CD4 TM peptides transition toward their native TM structure of a single-pass α-helix (Figure 2D). The CD4 TM peptide improved its helicity composition from 8% in Tris buffer to 73% in the presence of the POPC vesicles. EGFR also is depicted as gaining helicity content when exposed to vesicles, although more modestly from 11 to 44%. Meanwhile, the pre-existing α-helicity of the $E_3\alpha$ peptide (50% in Tris) is minimally altered by the introduction of POPC vesicles, confirming that the peptide has no significant interaction with the vesicles, as expected. Overall, these data reflect that the CD4 and EGFR TM peptides can be reconstituted into biomimetic membrane environments and, importantly, are able to achieve native-like secondary structure.

Functionalization of Tethered Bilayer Lipid Membranes with TM Peptides. Characterizing the structure of membrane proteins integrated into lipid bilayers requires a real-time assessment of TM peptide insertion and/or activity within an immobilized planar membrane. Ideal for monitoring interactions between soluble proteins and immobilized ligand at a two-dimensional surface, SPR was applied to detect the association of the biotinylated TM peptides to the planar membrane, and subsequent SA capture to the peptide–membrane surface.

POPC lipid bilayers were generated by the "rapid solvent exchange" technique to a SAM of thiolated lipids on a gold-coated SPR substrate (see the Materials and Methods for details). To establish that proper tBLM formation has been achieved, we used EIS measurements *in situ* with SPR (see the Supporting Information for details of EIS). After a stable baseline in the SPR response was observed, EGFR or CD4 peptide was incubated with the independent planar bilayer surfaces for 5–10 min, in the background of Tris buffer (again, with a volume fraction of 10% acetonitrile to limit peptide aggregation). Over this time, the SPR signal difference before and after TM peptide incubations is small, making it challenging to assess the amount of peptide that had associated with the bilayer (Figure S3). A possible interpretation of this result is that peptide insertion within the tBLM displaces an equivalent volume of phospholipids, minimizing the net change in associated biomass. The presence of acetonitrile in the Tris buffer (for solubilizing the hydrophobic peptides) may facilitate lipid displacement, because we have observed that acetonitrile has the potential to thin the membrane somewhat by solubilizing the phospholipids (Figure S3). Importantly, however, EIS measurements showed no increase in defects of the bilayers following peptide and acetonitrile exposure (Table S1). Meanwhile, surface aggregation of the peptides can be ruled out, because there are no indications of steady, irreversible accumulation of biomass from the SPR responses.

To demonstrate TM peptide incorporation within the POPC membrane, biotin-binding SA protein was incubated with the peptide–membrane surface. The N-terminal biotin tags of the TM peptides embedded within the bilayer should present high-affinity targets for SA (K_D < 1 pM),[42] thus resulting in a sharp and largely irreversible SPR response. Indeed, after either CD4 or EGFR peptide incubation, surface capture of SA was clearly observed by the resulting 11.8 and 16.5 pixel unit increases, respectively (Figure 3A). A conversion of the pixel changes to surface coverage translates to roughly 17 and 24% of the available planar space above the CD4- and EGFR-treated membranes as occupied by the captured SA (see the Materials and Methods for calculations). In contrast, exposure of SA to the neat tBLM or the $E_3\alpha$-treated membrane resulted in a minimal SPR response that completely reversed after buffer rinsing. Thus, SA does not participate in non-specific interactions with the tethered POPC membrane surface yet effectively associates with membranes activated by biotin-tagged peptides. Further, these results indicate that significant populations of the TM peptides are oriented in such a way as to sufficiently expose the biotin-terminated end of the peptide toward the outer membrane surface.

Alternatively, in an attempt to anchor a TM peptide within the tBLM in a uniformly oriented fashion, we preformed the EGFR–SA complex prior to POPC bilayer incubation. This would result in only the C-terminal end of the TM peptide free to insert into the tBLM surface, because the N-terminal end with the biotin tag would be affixed to the binding sites of SA. When protein association was monitored to the tBLM once again by SPR, it was evident that the TM peptide of the EGFR–SA complex anchored irreversibly into the membrane, producing a comparable level of protein binding as the unligated SA to the peptide–membrane surface (Figure 3B). The relatively slow kinetics observed for the association of the EGFR–SA complex suggests a different mechanism for

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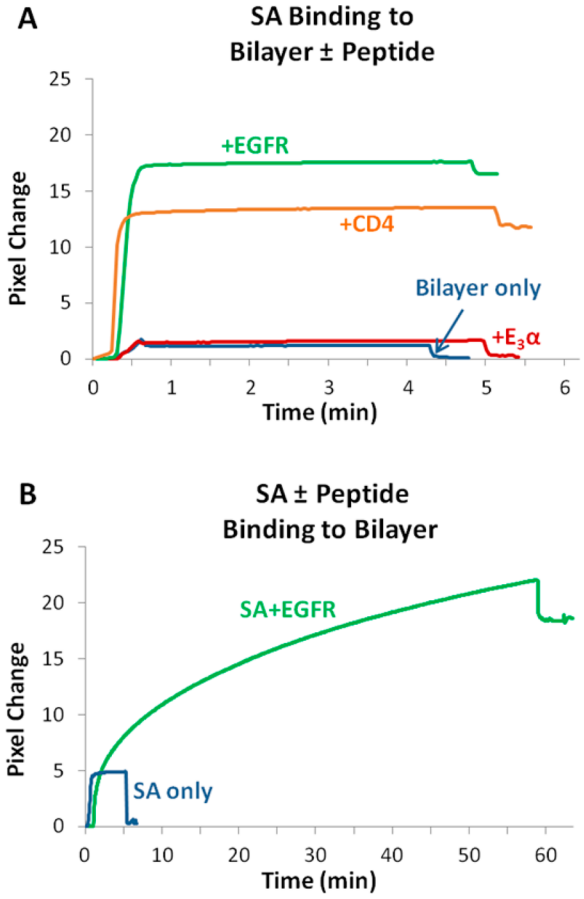

Figure 3. SA capture with planar bilayer systems activated by TM peptides. (A) Bilayers that have been pretreated with 5 μM peptide or no peptide (blue trace) are incubated with 5 μM SA for approximately 5 min before phosphate buffer rinse. (B) Pretreating the soluble SA with EGFR TM peptide (4:1 molar ratio of SA/EGFR) results in similar (albeit slower) capturing efficiency compared to a peptide-treated bilayer, demonstrating an alternative method for forming a peptide–SA complex at the bilayer surface.

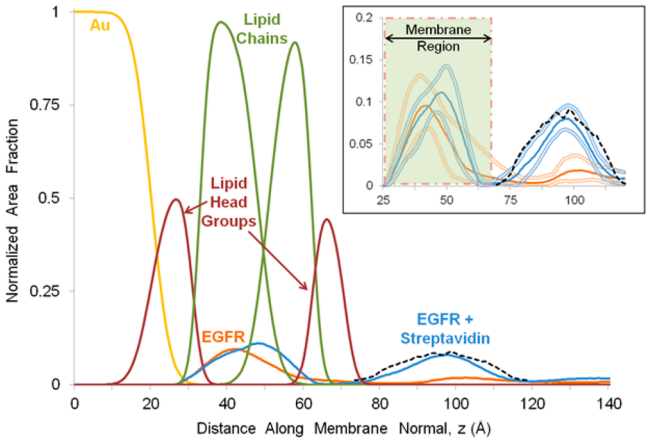

Figure 4. NR-derived profile of the tBLM, followed by incubation with the EGFR peptide and then capture of SA. The structure of the neat tBLM is shown as the area fraction distribution of the Au substrate (gold line), the lipid acyl chain (green line), and the headgroups (crimson line) of the inner and outer lipid leaflets. Subsequent profiles for the tBLM after protein addition were unchanged and, therefore, not depicted. The protein profiles after EGFR TM peptide incubation (orange line) followed by SA incubation (blue line) are depicted. An overlay of the SA profile calculated from an X-ray structure,[43] with one of the four biotin binding pocket interfaces aligned with the membrane surface, is also shown (black, dashed line). (Inset) Closer focus on the protein profiles, with 95% confidence bands indicated by colored double lines.

membrane insertion of the TM peptide when coupled with SA, as opposed to the tBLM functionalization by the free peptide. Nevertheless, both methods provide evidence that functionalized TM peptides possess the capability to couple soluble proteins, such as SA, to a planar membrane surface.

Structural Characterization of Peptide Insertion and SA Capture by NR. NR is well-suited for the characterization of planar lipid films, such as the tBLM system used in the SPR experiments, by providing one-dimensional (1D) information on the distribution of molecular groups in the direction normal to the membrane. Here, NR was used to resolve the profile of the EGFR TM peptide inserted in the tBLM and the subsequent capture of SA to the membrane surface. Statistically significant changes were observed in the NR spectra for each protein treatment of the tBLM for all three solvent contrast conditions measured (Figure S4). Figure 4 shows the distributions of the lipid molecular groups (acyl chains, green lines; headgroups, crimson lines) in terms of surface area fraction from measurements of the neat tBLM. The fit results indicate a well-formed bilayer of approximately 99% completeness. After EGFR TM peptide incubation with the tBLM surface, the spline model shows the addition of a protein distribution within the bilayer (orange line), which corresponds to an area fraction of roughly 10% (±3% confidence intervals). Protein material modeled beyond the membrane is negligible, within the uncertainty of the model. Thus, the NR data of the EGFR–tBLM surface suggests that the EGFR TM peptide inserts into the tBLM, spans the membrane, and remains stably embedded within the bilayer.

Next, we exposed the peptide–membrane surface to SA, to characterize the capturing potential and orientation of the biotinylated EGFR peptide within the planar bilayer. This resulted in a substantial change in the reflectivity profile beyond the bilayer and was modeled as an additional protein layer with an area fraction of 8% (±1% confidence intervals), accumulated on the membrane surface (blue line). Meanwhile, the protein spline peak within the dimensions of the bilayer profile, which accounts for the EGFR peptide, essentially stayed constant, within error, in overall area fraction. Thus, SA capture by the N-terminal biotin tag of the EGFR TM peptide results in exclusively peripheral membrane association. To reinforce the interpretation of the experimental spline model of a monomolecular layer of SA protein, a 1D protein profile was calculated from a high-resolution X-ray structure of tetrameric SA.[43] Strikingly, the alignment of two of its biotin-binding pockets along the membrane surface (black dashed line in Figure 4) matches closely to the dimensions calculated by the NR spline model. These results lend further credence to an interpretation of the data as a monomolecular layer of SA captured to the membrane surface by the functionalized EGFR TM peptide.

DISCUSSION

This work demonstrates a modular strategy for functionalizing lipid membranes, using scaffold TM peptides that possess the ability to spontaneously insert into the bilayer and present biochemically active tags at the surface. We began by

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attempting to anchor functionalized peptides mimicking TM domains of single-pass receptors within biomimetic lipid bilayers. First, solution-based assays of tryptophan fluorescence were performed to evaluate the efficiency of TM peptide partitioning within the lipid bilayer of POPC vesicles. Insertion into the vesicle bilayers measured by fluorescence of internal tryptophan (Trp) residues in the CD4 and EGFR TM peptide sequence was conclusive from the reproducible and drastic blue shifting of the fluorescence intensity maximum wavelength ($\Delta\lambda_{max}$). Distance-dependent bromine quenching on account of the 1:1 Br_2PC/POPC vesicles provided more precise detail of the penetration by the hydrophobic core of the TM peptides. The significant level of quenching imposed on the fluorescence of the EGFR TM peptide by the brominated lipids (>50% reduction in fluorescence) demonstrates efficient insertion within the vesicle bilayers. Because the bromine atoms of the three Br_2PC variants reside an estimated 6.5−11.0 Å from the membrane center,[40] it can be gleaned from the trends in the fluorescence quenching that the EGFR peptide penetrates the vesicle with its internal Trp residue approaching the bilayer center. This evokes an image of a substantial population of the TM peptides partitioning squarely through the bilayer of the vesicle. In comparison, the trend in the bromine quenching for the CD4 TM peptide reflects its Trp position to be further from the bilayer center. While it could be interpreted that the CD4 TM peptide may fail to fully partition the Br_2PC/POPC vesicle bilayer, the quenching effect can partially be explained by the off-centered placement of the Trp several residues more proximal to the C-terminal CD4 TM primary sequence, a consequence of making a chemically conservative substitution of the most internal phenylalanine residue (see the Materials and Methods). The positional difference between the CD4 and EGFR Trp residues is estimated at 5.3 Å along the membrane normal direction with respect to their backbone Cα atoms. However, the aromatic rings of the Trp side chains, from where the fluorescence originates, could be separated by more than 10 Å, depending upon the side-chain conformation of each peptide. Thus, the fluorescence results for CD4 may still report on a membrane-spanning TM peptide, particularly considering the complete lack of bromine quenching observed for the negative control $E_3\alpha$ peptide.

Assessing the α-helical structure of the TM peptides by CD confirmed that the peptides have a propensity to fold into native-like TM-spanning structures within the vesicle bilayers. This was particularly the case for the CD4 TM peptide, which increased by an average of 65% toward α-helical conformation when incorporated into the POPC lipid vesicles. The 73% helical structure of the CD4 peptide after POPC exposure is consistent with a peptide predominantly inserted in a TM orientation across the bilayer, especially when compared to the positive control $E_3\alpha$ peptide, whose Ala-rich sequence has an intrinsic proclivity for α-helical conformation.[27] Emphasizing this point, nearly the same CD profile was produced when the CD4 TM peptide was mixed with SDS detergent micelles (dashed line in Figure 2A). Similar DPC micelles were used to characterize the purely α-helical NMR structure of the CD4 TM domain,[41] strongly inferring that our CD4 TM peptide adopts a comparable α-helical structure in biomimetic POPC vesicles. Meanwhile, the more modest increase (33%) toward an α-helical structure by the EGFR TM peptide puts it at equivalent levels calculated for the positive control $E_3\alpha$ peptide. Notably, SDS micelles were more effective at facilitating greater α-helicity formation for the EGFR TM peptide (dashed line in Figure 2B), which NMR structures depict as predominantly α-helical in detergent micelles.[44] Why the EGFR TM peptide may adopt sub-optimal α-helical conformation compared to the CD4 TM peptide is a question that we cannot answer at this point. Still overall, the assays of the TM peptides with lipid vesicles demonstrated that the receptor TM domains were at least partially folded and embedded within a biomimetic membrane.

Next, we wanted to showcase the potential of these biotinylated TM peptides to anchor soluble proteins to a solid-supported tBLM. Biotin and SA are known to form one of the strongest non-covalent complexes in biochemistry and were used here to report on peptide−protein coupling in the context of our membrane system. The SPR results explicitly demonstrated that, after CD4 or EGFR TM peptide insertion, the bilayer was activated, capable of tightly and irrevocably anchoring SA to the membrane. Both the neat POPC membrane and the membrane after $E_3\alpha$ exposure showed negligible and immediately reversible changes in their SPR response, confirming the robust specificity of the SA protein interaction with the TM peptides. NR measurements, meanwhile, provided a coarse-grained structural picture of the EGFR TM peptide spanning the dimensions of the POPC lipid leaflets while also resolving a protein profile of a monomolecular layer of SA on the membrane surface that could be well-approximated from the SA crystal structure. The alignment of the X-ray structure such that the biotin-binding pockets are adjacent to the membrane surface also coincided with the shortest dimensions of the molecule along the membrane normal direction. Significant deviations from this orientation would result in a broader profile that would easily extend beyond the confidence bands determined by the free-form spline model (inset in Figure 4). Within the membrane, contributions from multiple molecular groups to the overall scattering length density (SLD) of that region significantly increase uncertainty in the TM profile, as indicated by the broader confidence bands. While this precludes a detailed modeling of TM orientation, the distribution of the peptide is clearly membrane-spanning both before and after SA capture.

Because of the nature of the spontaneous insertion of the TM peptides into both lipid vesicles and a tethered bilayer, the directionality of the peptides (i.e., oriented with biotin facing the bulk aqueous reservoir, versus facing toward the tethered substrate) cannot be controlled. In fact, the reflectivity data indicated an approximate 1:1 ratio of area fraction for EGFR peptide to captured SA, which translates to a 16:1 molar ratio (because EGFR is ≈3.6 kDa, while tetrameric SA is ≈53 kDa). This represents only a 7% capturing efficiency for the SA protein. A randomized orientation of the N-terminal biotin in the bilayer would account for a 50% reduction in the overall capture efficiency (assuming degeneracy of the two major "peptide-up" and "peptide-down" conformations). Further reduction in capture efficiency can be explained by even correctly oriented peptides having their biotin moiety partially submerged in the membrane headgroup region, sterically hindering the SA interaction. The results suggest design improvements, such as extending the linker beyond the four or five Lys residues of the EGFR and CDR peptides, respectively. In an attempt to circumvent these issues, we demonstrated that pre-forming the EGFR−SA complex before exposure to the tBLM could be an effective strategy for achieving a uniform orientation of peptide within the membrane. How to apply this strategy for anchoring TM

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peptides as a step toward reconstructing larger biosurface assemblies is still conceptually raw but has seemingly great potential applications.

A biologically relevant explanation for the poor capture efficiency of SA, as depicted by the NR data, is that the EGFR peptide may preferentially organize as dimers within the tethered bilayer. Indeed, the EGFR TM domain has been shown to exist as a dimer in biomimetic environments,[45] including in its NMR-derived structure,[44] as well as a native conformation for the TM domain in its active state for receptor signaling.[46] Dimerization of the TM peptide would severely limit the available docking space for a SA protein, which occupies ≈30 times larger surface area than the membrane-spanning peptide. In fact, the nearly 3-fold discrepancy between the surface coverage of sequestered SA determined by the SPR and NR data may be a result of the longer data acquisition time needed for NR experiments (approximately 6 h), which may allow for a higher percentage of dimer formation between the EGFR peptides. Resolving the mechanisms causing these discrepancies for some of our experimental outcomes is a technical challenge and more than likely mandates bringing other surface-based methodologies into the fold.

CONCLUSION

We characterized the structure and functionality of two TM peptides partitioned within fabricated biomimetic membrane surfaces and, in doing so, established practical groundwork for resolving more intricate biophysical properties of TM domains of proteins within a tBLM system. For example, alterative formulations of the tBLM (e.g., cholesterol, phosphatidylinositols, etc.) and mixtures of complementary receptor domains could induce observable and functionally pertinent changes in the properties of TM peptides. Also, complementary surface characterization techniques to SPR and NR, such as total internal reflection fluorescence (TIRF[47,48]) and atomic force microscopy (AFM[49]), would be amenable for additional structural perspectives on the membrane−peptide−SA complexed surface.

It is anticipated that more complex protein systems can cultivate from the TM peptide−membrane scaffolding that we have characterized here. Newly developing chemical and enzymatic ligation strategies will serve as a conduit to reach this significant milestone in engineering solid-supported membrane biosurfaces. For instance, ligation strategies using the sortase A transpeptidase from *Staphylococcus aureus* have made substantial progress in engineering hybridized peptides[50] and proteins,[51] including the linkage of globular proteins to artificial liposomes and neuron cell membranes.[52,53] Thus, it seems a lucrative endeavor to apply these types of ligation strategies to reassemble biosurfaces with intact receptor proteins and investigate their structures within the context of a native-like membrane environment.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.5b01990.

Supplemental methods, SPR sensorgrams of peptide incubations and corresponding EIS capacitance plots, circuit models, and fitted parameters, NR data (PDF)

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Notes

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